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Mesangial function and glomerular sclerosis in rats with aminonucleoside nephrosis

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Mesangial function and glomerular sclerosis in rats with aminonucleoside nephrosis. The possible relationship between mesangial dysfunction and development of glomerular sclerosis was studied in the puromycin aminonucleoside (PAN) model. Five male Wistar rats received repeated subcutaneous PAN injections; five controls received saline only. After 4 weeks the PAN rats were severely proteinuric (190 ± 80 mg/24 hr), and all rats were given colloidal carbon (CC) intravenously. At 5 months glomerular sclerosis was found in 7.6 ± 3.4% of the glomeruli of PAN rats; glomeruli of the controls were normal. Glomeruli of PAN rats contained significantly more CC than glomeruli of controls. Glomeruli with sclerosis contained significantly more CC than non-sclerotic glomeruli in the same kidneys. CC was preferentially localized *within* the sclerotic areas of the affected glomeruli. Since mesangial CC clearance from the mesangium did not change during chronic PAN treatment, we conclude that this preferential CC localization within the lesions is caused by an increased CC uptake shortly after injection in apparent vulnerable areas where sclerosis will develop subsequently. Cluster analysis showed a random distribution of lesions in the PAN glomeruli in concordance with the random localization of mesangial areas with dysfunction in this model. Similar to the remnant kidney model in PAN nephrosis the development of glomerular sclerosis may be related to "mesangial overloading."

Fonction mésangiale et sclérose glomérulaire chez des rats atteints de néphrose aux aminonucléosides. La relation possible entre le dysfonctionnement mésangial et le développement d'une sclérose glomérulaire a été étudié dans le modèle à la puromycine aminonucléoside (PAN). Cinq rats mâles Wistar ont recu des injections répétées sous-cutanées de PAN, et cinq contrôles ont recu du sérum salé seulement. Au bout de 4 semaines, les rats PAN étaient sévèrement protéinuriques (190 ± 80 mg/24 hr), et tous les rats ont recu du carbone colloïdal (CC) par voie intraveineuse. Au bout de 5 mois, une sclérose glomérulaire a été trouvée chez 7,6 ± 3,4% des glomérules des rats PAN; les glomérules des contrôles étaient normaux. Les glomérules des rats PAN contenaient significativement plus de CC que les glomérules des contrôles. Les glomérules avec sclérose contenaient significativement plus de CC que les glomérules non scléreux des mêmes reins. CC était localisé préférentiellement *dans* les zones scléreuses des glomérules atteints. Puisque la clearance mésangiale du CC à partir du mésangium ne changeait pas pendant un traitement chronique par le PAN, nous concluons que cette localisation préférentielle du CC dans les lésions est due à une augmentation de la captation du CC peu après l'injection par des zones en apparence vulnérables où la sclérose se développera ensuite. L'analyse des foyers a montré une distribution aléatoire des lésions dans les glomérules PAN, en accord avec la localisation aléatoire des zones mésangiales en dysfonctionnement dans ce modèle. Comme dans le modèle de rein restant, dans la néphrose au PAN le développement d'une sclérose glomérulaire pourrait être lié à une "surcharge mésangiale."

In rats glomerular lesions akin to focal and segmental glomerular hyalinosis and sclerosis (FSGHS) in humans may develop spontaneously [1, 2] after partial removal of functional kidney tissue [3] and after treatment with puromycin aminonucleoside (PAN) [4]. The first histologic change consists of a local deposition of amorphous eosinophilic material in the mesangium and subendothelium ("insudation") followed by mesangial matrix overproduction with capillary collapse and adhesions to the Bowman capsule ("sclerosis") [1]. The pathogenesis of FSGHS is as yet not clarified. It has been suggested that dysfunction and overload of the mesangium may lead to sclerosis and scarring [1, 2, 5]. After extreme renal ablation an increased mesangial accumulation of intravenously injected ferritin and rapid development of FSGHS were observed [6]. In uninephrectomized rats a preferential localization of injected colloidal carbon was found in those mesangial areas where sclerosis apparently developed at a later time suggesting a direct relationship between mesangial accumulation of circulating material and glomerular sclerosis [7]. In these so-called remnant kidney models the increased mesangial delivery of circulating substances and development of FSGHS is thought to be hemodynamically mediated [3, 6, 7].

The current study deals with the pathogenesis of FSGHS in the chronic PAN model of focal sclerosis. PAN nephrosis is characterized by mesangial dysfunction [5, 9]. In contrast to the remnant kidney models hemodynamic factors do not appear to play an important role [8, 9]. We present data suggesting that in chronic PAN nephrosis a locally increased mesangial uptake of circulating material may precede and induce FSGHS. Thus, although the remnant kidney on the one hand and the chronic PAN model on the other are clearly different with respect to many renal functional features, they share the development of FSGHS possibly due to mesangial dysfunction as a common quality.

Methods

Rationale of experimental design

Chronic PAN nephrosis was induced by repeated subcutaneous injections with PAN. The possibility of a direct relationship between the development of FSGHS lesions and locally increased mesangial activity was studied using colloidal carbon

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(CC) as a marker. CC was chosen since the particles are indigestible, taken up quickly by the mesangium and retained for months [10]. In experiment A CC was injected 4 weeks after the start of the PAN injections, when the animals were severely proteinuric and an increased mesangial functional activity could be expected. At this interval FSGHS lesions had not yet developed [5, 9]. The relationship between localization of CC and FSGHS was studied by light microscopy at 5 months when it was expected that FSGHS lesions had developed. If this development of the FSGHS lesions and their segmental character was related directly to local increased mesangial activity, we expected to find most remaining CC in the diseased glomeruli with a preferential localization of CC in FSGHS lesions. However, such a preferential localization might also be due to disturbed mesangial clearance although on the basis of experiments in the acute variant of the model this was considered unlikely [5, 9]. To check mesangial CC clearance during chronic PAN administration, in experiment B PAN injections were started *after* CC administration. After 5 weeks mesangial carbon content was compared to that of saline-injected controls.

In PAN nephrosis a striking increase in the mesangial accumulation of various exogenous tracers has been shown [5, 11]. In an ultrastructural analysis we observed an enhanced accumulation of intravenously injected CC and endogenous plasma proteins in randomly distributed segmental mesangial areas [9]. Therefore, if a relationship exists between increased mesangial uptake of circulating substances and development of FSGHS in PAN nephrosis, a random intraglomerular distribution of early FSGHS lesions should be found in contrast to the localization of injected CC and FSGHS lesions at the glomerular hilus after unilateral nephrectomy [1, 7].

In experiment C the intraglomerular distribution of FSGHS lesions in PAN nephrosis was therefore analyzed by a cluster method at the light microscopic level and compared to data obtained for FSGHS lesions induced by unilateral nephrectomy.

Experiment A

Ten male Wistar rats aged 3 months and weighing 180 to 220 g were used. They were fed a normal rat chow with a sodium content of 0.44% and a digestive protein content of 22% (Hope Farms, Inc, Woerden, The Netherlands) with free access to tap water. Five rats were injected subcutaneously with 2 mg of PAN (Sigma Chemical Company, St. Louis, Missouri, USA) per 100 g of body wt dissolved in 1 ml of saline weekly for 3 weeks and subsequently every other week [12]. The other rats received saline subcutaneously. Urinary protein excretion was measured by the biuret method in urine collected weekly by housing the rats in metabolic cages for 24 hr. During this time they had access to tap water only. Four weeks after the start of the PAN injections all animals received 30 mg of colloidal carbon (CC) per 100 g of body wt i.v. followed by 20 mg CC per 100 g at weeks 5 and 6 (colloidal carbon for biological use C11/1431[a], Günther Wagner, Hanover, Germany, containing 100 mg of CC/ml). The rats were killed 5 months after the start of the experiment. From both kidneys of each rat two tissue slices were fixed in 8% buffered formalin and embedded in glycolmethacrylate. From each tissue block two consecutive 2-mμ sections were cut. One set of sections was stained with periodic acid Schiff (PAS) for light microscopic examination (LM); the other set was left unstained and used for the measurement of the amount of mesangial carbon. The PAS-stained sections were screened at a magnification of $\times 250$ to determine the percentage of glomeruli showing FSGHS. The amount of mesangial carbon was assessed semiquantitatively with a modular scanning image analyzer (Optomax, Ealing Beck, Ltd, Whatford, England) as described in detail in earlier studies [7, 13]. Briefly, the method is a point counting method in which the total number of points covered by a contrasted area is counted and serves as a measure of the total area of carbon present in the glomerulus. In each unstained section 15 consecutive glomeruli sectioned through their largest or nearby largest diameter were measured moving through the cortex from surface to medulla and vice versa. In this way 60 glomeruli per rat were studied. Mean count number per glomerulus was taken as a measure of mesangial carbon content of that rat and the mean count of the five rats as the value for the group. For a more detailed analysis glomeruli were grouped in classes of increasing count number with a class range of 30 counts [7]. In the kidneys of the PAN-injected rats the distribution of 238 normal and 47 diseased glomeruli over the different count classes was also determined separately. In addition, PAS-stained sections were used to categorize diseased glomeruli according to the intraglomerular distribution of CC deposits as follows: A, almost all CC *within* the lesions; B, CC deposits both in sclerotic and non-sclerotic areas; C, CC mainly localized in nonsclerotic areas. A total of 140 glomeruli with FSGHS was analyzed.

Experiment B

Ten male Wistar rats were injected with CC during 3 weeks in a manner similar to experiment A. Five days after the last carbon injection blood samples were obtained and analyzed spectrophotometrically at a wavelength of 650 nm for the presence of circulating CC [10]. Subsequently, five rats received regular subcutaneous injections with PAN, the others with saline. The protocol of the subcutaneous injections was similar to that of experiment A. Proteinuria was measured weekly. All animals were killed 5 weeks after the first subcutaneous injection. Processing of kidney tissue and the semiquantitative measurement of mesangial carbon content were performed similarly to that of experiment A.

Experiment C

PAS-stained glycolmethacrylate sections of the kidneys of 15 PAN-treated rats (the animals of experiment A and PAN-treated animals used in other studies [12]) and of 17 unilaterally nephrectomized (UN) rats [7] were used for morphometric analysis. The distribution of early FSGHS lesions in the glomeruli was investigated by studying sections of glomeruli cut through their largest or nearby largest diameter and the vascular pole (afferent and/or efferent arteriole present). The "insudations" consisting of hyalin PAS-positive deposits in the mesangium and subendothelium were used as the hallmark of the early FSGHS lesion [1, 14]. The sections were screened at a magnification of $\times 375$ using a light microscope (Zeiss, Oberkochen, Federal Republic of Germany) equipped with a side tube attachment and a drawing prism. The image of a given glomerulus as viewed through the microscope was traced with

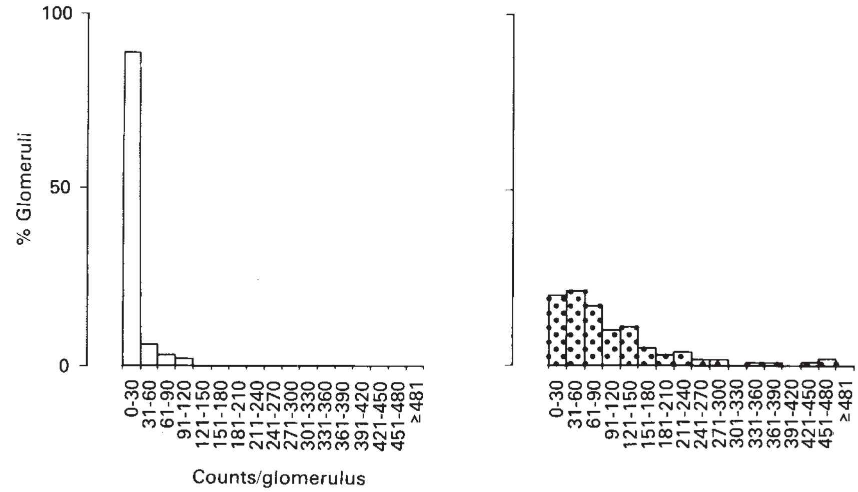

Fig. 1. *Frequency distribution of glomeruli of saline (□, N = 220) and PAN-treated (⊡, N = 285) rats at sacrifice (5 months) over numbers of counts per glomerulus.* Counts per glomerulus were grouped in classes of increasing count numbers with a class range of 30 counts. Carbon was injected 4 months before after severe proteinuria had developed in the PAN-treated rats.

a cursor along the Bowman capsule over the surface of a graphic tablet (Computex, GT 50/10, USA) connected to a Digital PDP 11/10 computer (Digital Equipment International, Galway, Ireland). The position of the glomerular hilus was indicated by starting with the cursor at that point. Subsequently, the insudation(s) were encircled within the given glomerulus. For analysis 81 glomeruli in the PAN and 104 glomeruli in the UN group were used. The lesions were projected in the mean glomerular cross sectional area of the given group. The mean free distance between the lesions within the given circle was calculated. Cluster analysis was performed as described by Schwarz and Exner [15].

Statistical analysis

Statistical analysis of the urinary protein data was performed using the two-sided Student's t test. The frequency distributions of glomerular percentages over the different classes of counts per glomerulus were skewed (see **Results**) and therefore compared using the nonparametric Mann-Whitney U test. A probability value less than 0.05 was regarded as significant. Where appropriate the standard deviation (SD) is given.

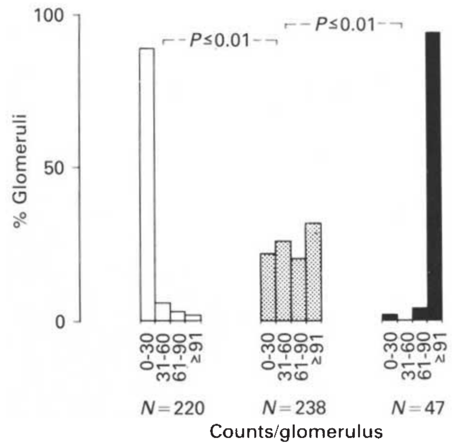

Fig. 2. *Frequency distribution of glomeruli of saline-injected (□) rats and normal (▨) and diseased (■) glomeruli of PAN rats over numbers of counts per glomerulus.* Counts per glomerulus were grouped in classes of increasing count numbers with a class range of 30 counts. P values refer to differences in frequency distribution (Mann-Whitney U test). Carbon was injected 4 months before sacrifice.

Results

Experiment A

In PAN rats proteinuria was detected 2 weeks after the first PAN injection. At week 4 urinary protein excretion in PAN rats was 190 ± 80 mg, in controls 18 ± 7 mg/24 hr ($P < 0.025$). Mean protein excretion during the total experimental period was 140 ± 62 mg/24 hr in PAN, and 20 ± 5 mg/24 hr in saline-injected rats ($P < 0.01$). (Mean protein excretion: the total of the results of all 24-hr protein excretion measurements divided by the number of collected specimens). At sacrifice 7.6 ± 3.4% of the glomeruli of the PAN-treated rats showed full-blown FSGHS lesions with "insudations" and sclerosis consisting of capillary wall wrinkling with collapse and adhesions to the Bowman capsule. No FSGHS lesions were found in the saline-injected group.

The mean carbon content of the glomeruli in the PAN rats was 111.2 counts and in the saline-injected group 14.2 counts per glomerulus. Figure 1 shows the frequency distribution of the glomeruli of the PAN and saline-injected rats in the different classes of counts per glomerulus. In the saline group 89% of the glomeruli belonged to the lowest class and none scored higher than 91 to 120 counts. In the PAN group a significantly higher percentage of glomeruli showed mesangial carbon in the class of 121 to 150 counts and over ($P < 0.01$, Mann-Whitney U test). Figure 2 shows a comparison of the frequency distribution of carbon content of normal glomeruli of the saline-injected rats and histologically normal and diseased glomeruli of the PAN-injected rats. In the group of diseased glomeruli a significantly higher percentage belonged to the count class of 91 and over as compared to the group of normal glomeruli in the same kidneys and to the group of glomeruli of the saline-injected rats (Mann-Whitney U test). Compared to the glomeruli of the saline-injected rats a significantly higher percentage of normal glomeruli of the PAN rats belonged to the count class of 31 to 60, 61 to 90, and 91 and over (Mann-Whitney U test). Mean

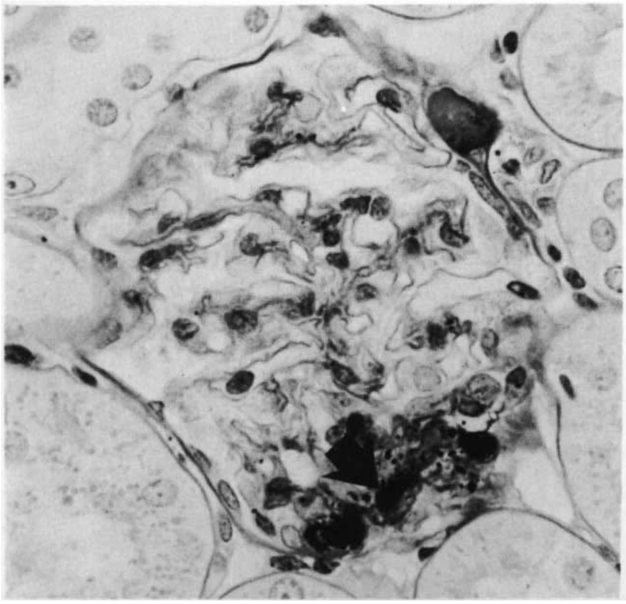

Fig. 3. *Representative glomerulus of a PAN-treated rat of experiment A.* Large accumulations of carbon are present within an area with sclerosis (*Arrow*). (PAS, ×250)

counts for the normal glomeruli of the PAN rats was 89.9 counts and for the diseased glomeruli 271.8 counts per glomerulus.

Light microscopic analysis of the intraglomerular localization of the mesangial CC deposits revealed that in 85.3 ± 3.7% of the glomeruli with FSGHS almost all CC was localized *within* the lesions, in 13.8 ± 4.6% of the glomeruli CC deposits were found both within sclerotic and non-sclerotic areas, and in 0.9 ± 1.0% of the glomeruli most CC was present in nonsclerotic areas. In addition to the full-blown FSGHS lesions many glomeruli showed areas with fresh insudations. Figure 3 shows a representative diseased glomerulus of a PAN-treated rat.

Experiment B

In none of the rats CC was detectable in the circulation 5 days after the last injection. During the period of PAN or saline injections, mean protein excretion was 149 ± 98 mg in the PAN and 17 ± 3 mg/24 hr in the saline group ($P < 0.01$). When sacrificed at 5 weeks FSGHS lesions were found neither in the PAN nor in the saline-injected rats. Mean carbon count was 62.0 in the PAN and 67.7 counts per glomerulus in the saline group. The distribution of the glomeruli of both groups over the different count classes did not differ significantly (Fig. 4).

Experiment C

In PAN rats FSGHS lesions were observed throughout the whole glomerular cross-sectional area without a preferential localization. Cluster analysis showed a nearly random distribution of the lesions occupying approximately 100% of the glomerular cross-sectional area. In the UN rats the lesions were localized as a cluster in 29% of the total glomerular area adjoining the glomerular hilus. Figure 5 shows the distribution of the FSGHS lesions within the glomeruli of both models. All lesions were projected on the half of the mean glomerular cross-sectional area. To join both parts for the figure the calculated mean glomerular cross-sectional area of the PAN rats was enlarged proportionally to reach the value of that of the UN rats.

Discussion

The current study indicates that the development of FSGHS in chronic PAN nephrosis is related to areas with an increased mesangial accumulation of a tracer injected at a moment when these lesions did not yet exist. The results of our experiments suggest that, as in unilateral nephrectomy [7], the development of focal sclerosis is related to disturbed mesangial function. The local accumulation of remaining CC in the lesions is most likely due to an increased mesangial CC *uptake* shortly after injection of the tracer when the PAN animals were clearly nephrotic rather than to a disturbed clearance. It has been shown that uptake of exogenous tracers by the mesangium is increased in the acute PAN model whereas clearance of these tracers from the mesangium is not changed [5, 9, 11]. The results of experiment B confirm this for the chronic model although it cannot be completely excluded that prolonged PAN administration (that is, longer than 5 weeks after the CC injections) may impair CC egress from the mesangium. Earlier data showed that the presence of CC per se does not induce sclerosis [7]. Velosa et al showed accumulation and persistence of intravenously injected protein aggregates in the glomerular areas with sclerosis that developed in chronic PAN nephrosis [16]. Because these aggregated proteins were given at the time the lesions were already present, these findings may be explained by local mesangial dysfunction or increased trapping in areas structurally changed by sclerosis. In the present study it is very unlikely that glomerlar lesions were present at the time of the tracer injections since in experiment B the glomeruli of the nephrotic rats were histologically normal after 5 weeks of PAN treatment. Moreover, as no carbon could be detected in the circulation 5 days after the last CC injection in experiment B, it is also unlikely that the presence of colloidal carbon in the lesions in experiment A was the result of uptake from the circulation in later phases of the experiment.

The reason for the increased mesangial activity in PAN nephrosis is not entirely clear. Hemodynamic changes do not appear to play an important role in view of the considerable decrease of glomerular capillary plasma flow rate and glomerular filtration rate with even a slight fall in single nephron filtration fraction [8]. In an earlier study we found a widening of the extracellular channels in segmental mesangial areas with increased accumulation of endogenous plasma proteins and injected CC [9]. These changes are possibly due to a direct toxic effect of PAN on the kidney [5]. In the chronic PAN model the vulnerable mesangial areas may be further damaged with a continuous exposure of the mesangial structures to larger quantities of circulating substances. In the course of time additional mesangial regions appear to be affected as well in view of the presence of fresh insudations not labeled with CC.

Although the development of FSGHS seems to be related to mesangial functional disturbance in the unilateral nephrectomy model as well as in the PAN model, the cause of this disturbance seems to be different. This can be concluded from the differences in localization of the early FSGHS lesions in these two models. In the UN animals the FSGHS lesions were clustered at the glomerular hilus confirming earlier observations

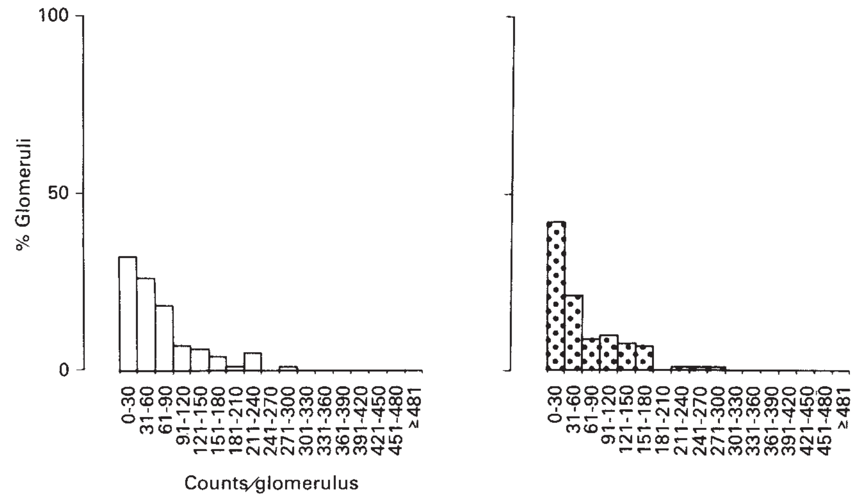

Fig. 4. *Frequency distribution of glomeruli of carbon-injected rats over numbers of counts per glomerulus after 5 weeks of saline* (□, *N* = 84) *or PAN* (▣, *N* = 90) *injections.* Counts per glomerulus were grouped in classes of increasing count numbers with a class range of 30 counts.

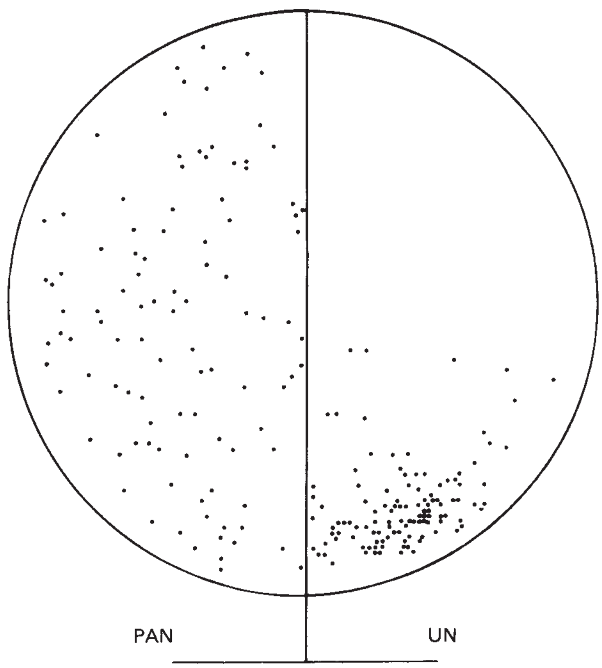

Fig. 5. *Intraglomerular distribution of FSGHS lesions in the chronic PAN* (left) *and unilateral nephrectomy* (UN, right) *model of focal sclerosis.*

in which both the FSGHS lesions and the increased mesangial CC uptake were localized at the vascular pole [1, 7].

Recently, much evidence has been provided that hemodynamic changes are an important factor in the pathogenesis of FSGHS developing after ablation of renal mass. After unilateral nephrectomy and five-sixths infarction of the remaining kidney, micropuncture studies showed a marked increase in the single nephron glomerular filtration rate of the remaining nephrons due to increase of glomerular plasma flow rate and mean glomerular transcapillary hydraulic pressure difference (that is, glomerular hyperperfusion and hypertension) [3]. In the same remnant kidney model Olson et al [6] observed a greatly increased deposition of intravenously injected ferritin in the mesangium with rapid development of FSGHS. A low protein diet prevented both hemodynamic changes, increased mesangial ferritin accumulation, and structural abnormalities [3, 6]. The nearly random distribution of the lesions in PAN-treated rats is quite compatible with a direct toxic influence as proposed in an earlier publication [9]. Thus, although the remnant kidney on the one hand and chronic PAN nephrosis on the other are clearly different experiment models in respect to renal functional characteristics, they share increased mesangial tracer accumulation and development of segmental sclerosis as common features.

The histologic lesions resulting in FSGHS start in the mesangium with segmental depositions of immunoglobulins M and G, complement, fibrin-related antigens, and lipids [1, 2, 7, 14, 16]. In view of these data many authors have attributed the pathogenesis of glomerular sclerosis to "mesangial overloading" [1, 2, 5]. Our results agree with this suggestion although it should be emphasized that the mesangial uptake and processing of inert colloidal carbon particles differ from that of endogenous biologically degradable substances [5, 10, 17]. However, a causal relationship between "mesangial overloading" and development of FSGHS cannot definitely be established on the basis of our results. So far, neither the character of the insulting substances nor the mechanism by which mesangial sclerosis takes place are specified precisely. Recently, emphasis has been put on the deposition of lipids as a possible contributing factor [7, 12, 18].

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